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Epstein-Barr Virus in Pediatric Hodgkin Disease: Age and Histiotype Are more Predictive Than Geographic Region

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Epidemiologic studies have implicated Epstein-Barr virus (EBV) in the great majority (80%–100%) of Hodgkin disease (HD) cases in South American countries, versus only 30%–40% in the United States and other industrialized countries. Other EBV-related malignancies are known to be geographically localized, including nasopharyngeal carcinoma in south China and Burkitt lymphoma in equatorial Africa. Some studies, however, have suggested that age and histiotype, rather than geographic region, are the major determinants of the association between EBV and HD. To further characterize this relationship in children, we matched 26 cases of pediatric Hodgkin disease from south Brazil and 26 cases from the U.S. for histiotype and age. The Brazilian children (22 males, 4 females) had a median age of 9 years, while the median age of the U.S. group (11 males, 15 females) was 7.5 years. Formalin-fixed, paraffin-embedded biopsy material was examined for EBV early RNA1 (EBER1) expression by *in situ* hybridization. This antigen was detected solely in Reed-Sternberg cells or their variants in positive samples. The same proportion of cases was positive (15/26 or 58%) in both groups of children. After adjustment for histiotype and age, the association between EBV and HD remained independent of geographic location, but was more frequent in children aged ≤10 years at diagnosis. These findings support the multiple-etiology hypothesis for Hodgkin disease. **Med. Pediatr. Oncol. 28:248–254.**

INTRODUCTION

Hodgkin disease (HD) appears to comprise several distinct histologic, etiologic, and epidemiologic subgroups [1–3]. Children and adolescents with this disease have an excellent prognosis, and the disease-free survival rate approaches 90% [4]. However treatment of HD can cause severe long-term sequelae, especially in children [5,6]. If more meaningful prognostic factors can be identified, it may be possible to tailor therapy more closely to individual features in this disease.

The relationship between HD and Epstein-Barr virus (EBV) is well established: a past history of infectious mononucleosis increases the risk of HD [7–12]; anti-EBV titers are elevated prior to the diagnosis of HD [13]; EBV genomic DNA is present in tumor tissue [14–17]; and viral RNA is detected in Reed-Sternberg (RS) cells and their variants [18–20]. The link between EBV and HD may help to identify patients with distinct clinical and biologic features.

Previous studies of the demographic characteristics of EBV-positive pediatric HD have yielded inconclusive results [21,22]. However, sensitive molecular testing can now improve assessment of these cases. To clarify the clinical, histologic, and epidemiologic correlates of EBV-positive pediatric HD, we used in situ hybridization [18,23] to detect EBV in paraffin-embedded tumor tissues from patients in southern Brazil and the United States (St. Jude Children's Research Hospital), matched for age at diagnosis and histologic subtype.

MATERIALS AND METHODS

Patients and Tissues

We studied formalin-fixed, paraffin-embedded diagnostic biopsy specimens from 26 patients under 21 years of age treated for HD at Hospital de Clinicas, in Curitiba, southern Brazil. These specimens were matched for histologic subtype, and as closely as possible for age, with

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TABLE I. Clinical Characteristics of Pediatric Hodgkin Disease Cases by Geographic Location

Clinical features	Brazil	U.S.A.
Age at diagnosis (years)		
Range	4–21	5–18
Median	9	7.5
Sex		
Male	22	11
Female	4	15
Clinical stage[a]		
Limited (I-II)	8	9
Advanced (III-IV)	17	17
Histologic Subtype		
Nodular sclerosis	6	6
Mixed cellularity	17	17
Lymphocyte predominance	1	1
Lymphocyte depletion	2	2

[a]Data on clinical stage not available for one Brazilian patient.

specimens from 26 patients treated at St. Jude Children's Research Hospital (SJCRH). Diagnosis and histopathologic classification of each case was confirmed by a pathologist (JJJ) in accordance with standard criteria [24] and without knowledge of clinical or experimental data.

The patients treated in Brazil were all native to that region, while none of the patients treated at SJCRH was of South or Central American extraction. All cases were HIV-negative. The clinical characteristics of both groups are outlined in Table I. Except for male predominance in the Brazilian group, there were no significant differences in the presenting clinical features of the two groups.

In Situ Hybridization Studies

Digoxigenin-labeled riboprobes specific for EBV early RNA1 (EBER1) were prepared as described previously [18,25]. Briefly, sense (complementary) and antisense riboprobes were generated by transcribing cloned DNA sequences using T7 and SP6 RNA polymerase, respectively, in the presence of digoxigenin-labeled uridine triphosphate as per manufacturer's instructions (Boehringer-Mannheim, Indianapolis, IN). Antisense probes to EBER1 recognize a nonpolyadenylated pol III transcript characteristic of latent EBV infection [26]. A sense probe to EBER1 served as a control for nonspecific hybridization.

Biopsy specimens were routinely fixed, processed and paraffin-embedded. Tissue sections were mounted on silane-coated glass slides and stored at room temperature. The sections were deparaffinized with xylene for 10 minutes and rehydrated in serial graded ethanol washes (100%, 90%, 70%, 50%, and 30%) and phosphate-buffered saline (PBS). After digestion with proteinase K (10 μg/mL in 100 mmol/L TRIS, 50 mmol/L ethythenediaminotetracetate [EDTA], 2 mmol/L calcium chloride) for 30 minutes at 37°C, the sections were rinsed in water and PBS with 0.2% glycine. They were then covered with 200 μL of prehybridization solution (50% formamide, 5× salted sodium citrate [SSC], 250 μg denatured salmon sperm DNA, 250 μg/mL yeast t-RNA, and 4 mmol/L EDTA). Coverslips were applied and the slides were placed in a humid chamber at 49°C for 3 hours. Slides were then dehydrated in serial graded ethanol washes (70%, 90%, 100%) for 2 minutes each. Up to this point, all solutions and glassware were handled in ribonuclease-free conditions.

Sections were hybridized to 1 μL of riboprobe (10 ng) in 25 μL of hybridization solution. Coverslips were placed over the sections and sealed with rubber cement. The slides were kept at 49°C overnight. After hybridization, coverslips were removed carefully and the tissue sections were washed at 43°C with frequent changes of 2 × SSC for 30 minutes, 0.2 × SSC for 15 minutes, and 0.1 × SSC for 15 minutes. The slides were agitated for 1 minute in buffer 1 (100 mmol/L TRIS, 150 mmol/L NaCl, pH 7.5) then blocked in 0.5% blocking reagent in buffer 1 at room temperature (RT) for 30 minutes. To identify the digoxigenin-labeled cells, sections were incubated with a 1:2000 dilution of antidigoxigenin antibody conjugate (Boehringer Mannheim) in buffer 1 for 30 minutes at RT. After washing with buffer 1 for 30 minutes at RT, the slides were equilibrated in buffer 3 (100 mmol/L TRIS pH 9.5, 100 mmol/L NaCl, 50 mmol/L $MgCl_2$) for 2 minutes. Adequate color development was achieved after treating with color solution (337.5 μg/mL nitroblue tetrazolium salt [NBT]), 175 μg/ml X-phosphate in buffer 3) for 3 hours in the dark. The reaction was stopped by washing slides for 5 minutes in buffer 4 (100 mmol/L TRIS pH 8; 1 mmol/L EDTA). When dry, slides were counterstained with aqueous nuclear fast red dye for 5 minutes, washed with water, and coverslipped using Permount (Fisher Scientific, Fairlawn, NJ).

Sections from formalin-fixed paraffin-embedded Akata cells (EBV-infected Burkitt lymphoma cell line) [27] and BL2 cells (EBV-negative Burkitt lymphoma cell line) were used as positive and negative controls, respectively. EBER1 staining was interpreted without knowledge of clinical data or other experimental results.

Statistical Analysis

The effect of geographic location was analyzed using the exact two-sided Mantel-Haenszel test in the Brazilian cohort, the relationship of age, subtype, sex, and stage to the frequency of EBV infection was tested by the two-sided Fisher's exact test. Since the patients from SJCRH were not randomly selected, the above statistical tests were not appropriate for that group. The relationship between EBV positivity and clinical variables of the SJCRH patients is reported descriptively.

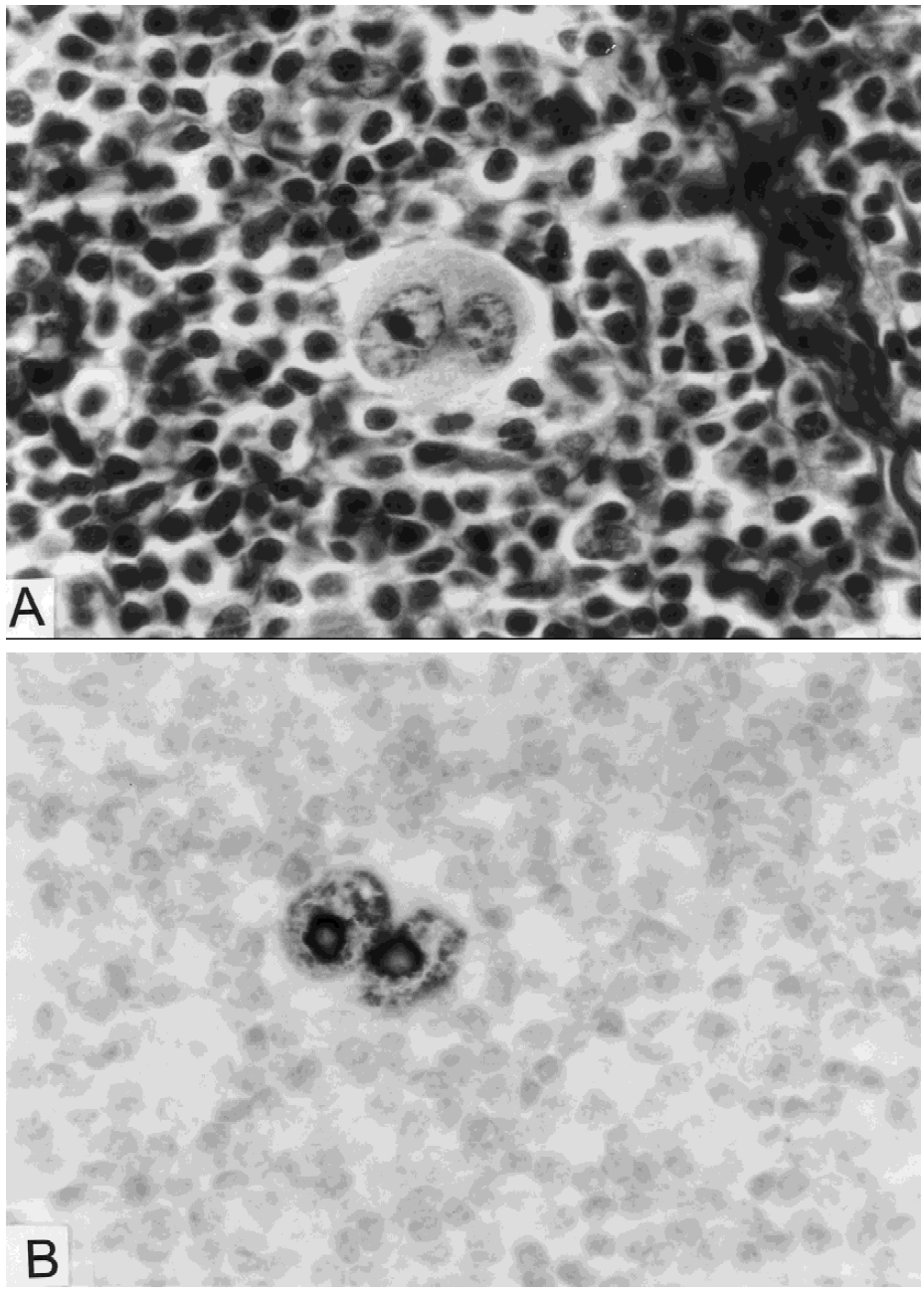

Fig. 1. Sections of lymph node from a child with Hodgkin disease show (A) Reed-Sternberg cell (H & E stain, original magnification 200×), and (B) an intense nuclear EBER1 hybridization signal in a Reed-Sternberg cell.

RESULTS

Detection of EBV Early RNA1 by in Situ Hybridization

EBER1 transcripts were identified in 30 of 52 cases (58%). In all 30 cases, positive signals were restricted to RS cells and their variants, as defined by large nuclei with prominent nucleoli (Fig. 1A). The EBER1 signal was apparent in the nuclei but not in the nucleoli (Fig. 1B). Sense probe to EBER1, which served as a control of nonspecific hybridization, was negative in all cases, including Akata EBV-positive control cells (Fig. 2).

Geographic Location

There was no significant geographic difference in the incidence of EBV. EBER1 transcripts were detected in the RS cells of 15 of 26 (58%) cases in each group (Table II). Geographic location was unrelated to the age and histiotype of patients with EBER1-positive tumors

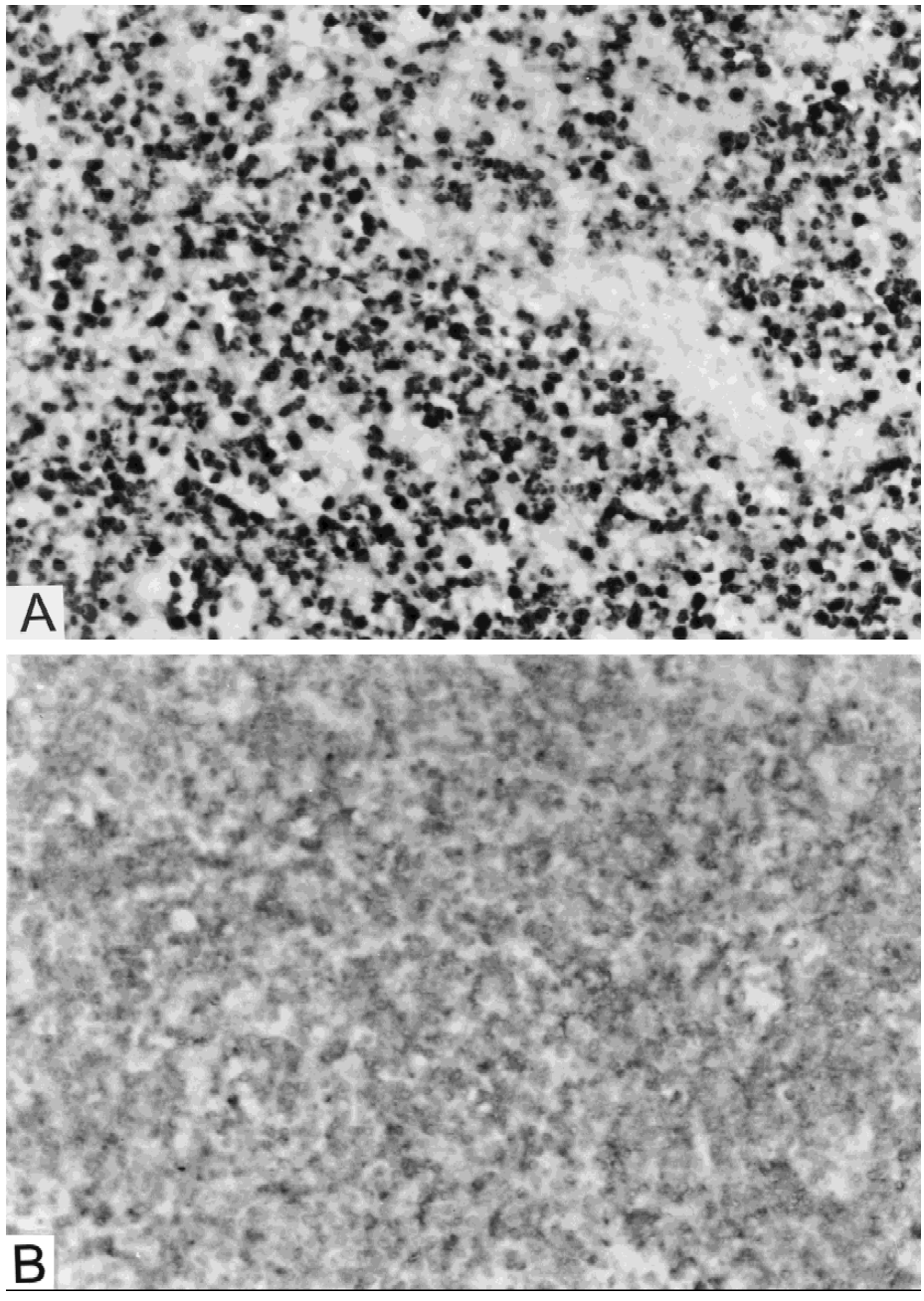

Fig. 2. EBV RNA expression in control Akata cells detected by *in situ* hybridization with (A) an EBER-1-specific antisense probe (positive control), and (B) an EBER-1-specific sense probe (negative control).

(Table II) ($p = 1$). Thus, the frequency of EBV positivity by age and histologic subgroups was similar in the two cohorts.

EBV Association by Histiotype

In the Brazilian children, EBV was detected most frequently in mixed cellularity (MC) HD cases (10 of 17; 59%), but also in 3 of 6 of the nodular sclerosis cases (59% vs. 50%) ($p = 0.71$) There were too few cases (n = 3) representing other histologic subtypes for meaningful statistical analysis. Among the U.S. group, 12 of 17 (71%) mixed cellularity and 2 of 6 (33%) nodular sclerosis cases were EBER-1 positive.

EBV Association by Age

Evidence of EBV was strikingly associated with age ≤ 10 years (Table III). Among the Brazilian patients 10 years of age or younger, 14 of 19 cases (74%) were EBV-associated, while only one of seven (15%) was EBER1 positive in the older group ($p = 0.02$). Similarly,

TABLE II. Distribution of EBER-1 Positive Cases by Geographic Location and Histologic Subtype

HD subtype	Brazil No. positive/total	U.S.A. No. positive/total	Total
Nodular sclerosis	3/6	2/6	5/12 (42%)
Mixed cellularity	10/17	12/17	22/34 (65%)
Lymphocyte predominance	0/1	0/1	0/2 (0%)
Lymphocyte depletion	2/2	1/2	3/4 (75%)
Total	15/26 (58%)	15/26 (58%)	30/52 (58%)

TABLE III. Distribution of EBER-1 Positive Cases by Geographic Location and Age at Diagnosis

Age (yr)	Brazil No. Positive/Total	U.S.A. No. Positive/Total
⩽10	14/19 (74%)	13/17 (76%)
⩾10	1/7 (15%)	2/9 (22%)

13 of 17 (76%) of the SJCRH cases ≤ 10 yrs of age at diagnosis were EBER1 positive, versus 2 of 9 (22%) children who were older.

EBV Association by Gender and Stage

In the Brazilian children, we found no statistically significant difference between males and females in the frequency of EBV positivity ($p = 0.61$). Similarly, no significant relationship was found between EBER1 positivity and clinical stage (limited vs. advanced) in the Brazilian cases ($p = 0.38$).

EBER1 was detected in 6 of 11 (55%) males from SJCRH and in 9 of 15 (65%) females. Nine of 17 (53%) advanced-stage U.S. cases were EBV positive, as compared to 6 of 9 (67%) limited-stage cases.

DISCUSSION

In this matched-cohort study, evidence of EBV in Reed-Sternberg cells was strongly associated with age below 10 years at diagnosis, rather than with patients' geographic origin. These results are consistent with previous reports that HD in children below age 10 years is clearly EBV-associated [22,28,29], and accounts in part for the much higher incidence of EBV-associated HD reported in South America, where patients tend to be younger. The distribution of EBV-positive cases by age and histologic subset was similar in the two geographic cohorts. Studies that match subjects for these two variables may help to illuminate the apparent differences between developing and industrialized countries in the frequency of EBV association with HD.

Previous attempts to characterize EBV-positive HD have yielded inconclusive results. In one study [21] of children age <14 years from Honduras and the United States, both geographic region (Honduras) and histiotype (mixed cellularity) were associated with EBV. However, since a third of the EBV-positive patients from the U.S. were of Hispanic origin, the sample may not have accurately reflected the average ethnic and socioeconomic characteristics of an industrialized country. Armstrong et al. found EBV more frequently in cases from Brazil or Saudi Arabia than in those from the United Kingdom, but the absence of a statistically significant difference suggested that factors other than geographic region were responsible [22]. In their study, both age below 10 years and mixed cellularity histiotype were associated with EBV. However, younger patients with mixed cellularity histiotype were under-represented in the UK group. Because of the potential confounding effects of factors not controlled in previous analyses, it has been difficult to interpret these studies. Alternatively, the small sample size may have contributed to the lack of difference among these geographic regions [30]. In Latin America, children with HD typically have a male predominance, young median age at diagnosis, and mixed cellularity histiotype [31]. By contrast, the clinical and laboratory characteristics of the SJCRH patients were typical of those seen in the U.S. and other industrialized countries [32,33].

On the basis of several factors including age, histologic subtype, and geographic incidence, MacMahon proposed that HD may represent more than one disease [1,34]. Recent studies in adult HD have supported the ''two-disease'' hypothesis [35], but little information is available for the pediatric age group. Our study clearly shows that the distribution of EBV-associated HD in children is not random. In children ten years old or younger, HD is predominantly an EBV-associated disease in both the American and Brazilian cohorts. In contrast, the association is much less frequent among older children from the two geographic regions. In addition, EBV was more frequently detected in mixed cellularity than in nodular sclerosis subtypes, consistent with several previous reports [32,36]. These findings, coupled with recent evidence suggesting genetic susceptibility to the young adult form of HD [36] and the previously documented risk of acquired HD acquisition among woodworkers [38] and after tonsillectomy and appendectomy [39,40], further support the multiple etiology hypothesis proposed by MacMahon.

Although recent studies have shown that EBV has no prognostic significance in adult HD [41–43], the greater frequency of EBV in HD among children age 10 or younger, as shown in this study, could facilitate prospective investigation of the relationship between EBV and clinical outcome in the pediatric population.

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